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TECHNICAL NOTE

A method for superfusion of the isolated perfused tubule

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The isolated perfused tubule technique has become a standard tool for studying renal function [1–3]. It has been established that essentially all segments of the mammalian nephron can be dissected from the kidney parenchyma and studied in vitro under controlled conditions [3]. The method has been primarily applied to defining transport properties of individual nephron segments. Another type of potential application of this technique is the study of synthesis and release of hormones or autocoids by individual nephron segments. This type of application requires a method for making repeat quantitative bath collections. Standard methods exist for collecting tubular perfusate [1, 2], and a method has been developed for collection of tubular reabsorbate [4], but standard methods are not available for continuous, quantitative bath collection. Interruption of bath flow to allow accumulation of a secreted product interrupts continuous delivery of fresh bath fluid to the tubule and results in uneven mixing. Bath changes are associated with mechanical disturbance to the tubule and can result in alterations in perfusion dynamics. The relatively large volume of the standard bath chamber results in dilution of any secreted product, while reduction in chamber size increases the difficulty of initiating perfusion.

In the present report we describe a technique for superfusion of the isolated perfused tubule. The method was developed to study the effect on renin secretion of changing fluid composition at the macula densa [5], but is potentially applicable to other nephron segments and other secreted products. It permits continuous quantitative collection of the fluid bathing the perfused tubule with minimal mechanical disturbance of the tubule.

Methods

The method employs standard isolated perfused tubule pipets, with the addition of an outer pipet which serves as a superfusion chamber. The pipets are illustrated schematically in Figure 1. The superfusion pipet (number 1) is made out of flint glass tubing (i.d. 3.5 mm, o.d. 5 mm) which is drawn on a vertical pipet puller (Stoelting, Chicago, Illinois, USA) and cut with a diamond at a diameter of 5 mm. The tip is fire-polished and siliconized. The pipets used for cannulation and perfusion of the tubule were prepared according to standard techniques [2] with the minor modification that the holding pipet had two inner constrictions (Fig. 1) approximately 400 μ apart; addition of the second constriction was found to assist in centering of the perfusion pipet [6]. The holding pipet had an inner diameter of 32 to 38 μ, with constrictions of approximately 25 μ. The superfusion pipet is mounted with a gasket in the front holder of a Greger-Hampel perfusion apparatus (Hampel, Frankfurt, W. Germany) [7] on a track which is movable independently of the inner concentric holding and perfusion pipets. The superfusate is delivered through a drawn polyethylene catheter to a port on the holder. The superfusion is driven by an infusion pump (Razel Scientific Inst, Stamford, Connecticut, USA) mounted with a 100 μl gastight glass syringe (Hamilton Instruments, Reno, Nevada, USA).

The three pipets were mounted on the track system and centered prior to use. The holding pipet was mounted first; it was moved as far back as possible. The superfusion pipet was then introduced into its holder, and the holding pipet advanced and centered by the centering screws on the superfusion pipet holder. The perfusion pipet was then introduced from the back and centering adjusted with the holding pipet centering screws. A standard isolated perfused tubule bath chamber (ITM, San Antonio, Texas, USA) was used. It is a thermoregulated lucite block heated by a built-in coiled stainless steel wire attached to a constant voltage source and governed by the sensor of a temperature controller (Yellow Springs Instruments Co., Yellow Springs, Ohio, USA). The total bath volume is 1 ml.

Slices were cut from the kidney of New Zealand white rabbits (0.8 to 1.2 kg) and immersed in ice-cold Krebs-bicarbonate solution containing 2% fetal calf serum. An individual juxtaglomerular apparatus (JGA), consisting of portions of the thick ascending limb of Henle (TAL), macula densa, and early distal convoluted tubule (DCT) with adherent glomerulus and short fragments of arteriole, was microdissected from the cortical portion of a medullary rays. Several thick ascending limbs were frequently adherent to a single glomerulus. The macula densa is not clearly visible at magnifications practical for dissection. Several landmarks were found useful in distinguishing the thick ascending limb with the macula densa from the other adherent TAL segments: the transition from TAL to distal convoluted tubule could usually be identified within 100 μ of the point of glomerular adherence [8]. A characteristic widening and bending of the tubule at the macula densa was apparent in most specimens.

The JGA was transferred to the bath chamber mounted on an inverted microscope (Olympus IMT-2). The thick ascending limb of Henle was cannulated and perfused, and the distal end was left free. A few specimens were perfused, in a retrograde manner. The perfused specimen was lifted free of the bottom of

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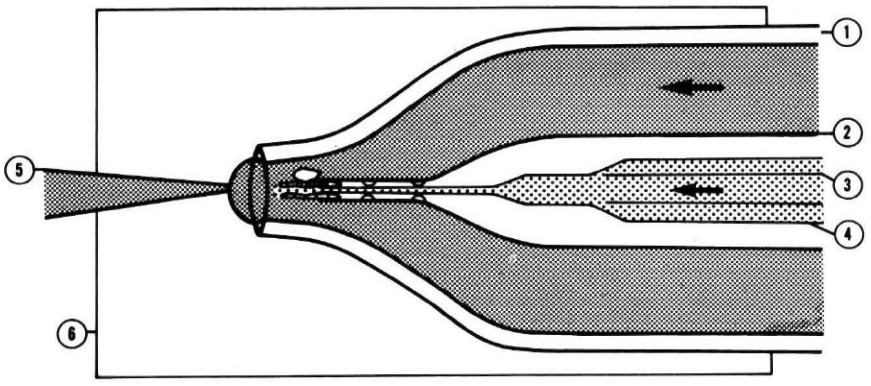

Fig. 1. *Schematic illustration of the method for superfusion of the isolated perfused tubule.* An outer pipet (1) serves as a superfusion chamber. It surrounds the standard isolated perfused tubule pipets, (2) the holding pipet, and (4) the perfusion pipet. Exchange tubing (3), inserted into the perfusion pipet is used for replacement of tubular perfusate. The bath chamber (6) is filled with mineral oil. The superfusate flows continuously and droplets collect at the tip of the superfusion pipet. These are collected at timed intervals (pipet 5). Pipets are not drawn to scale.

the chamber, and the tubule examined with differential interference contrast optics to verify the presence of the macula densa. It was sometimes necessary to rotate the specimen with a second pipet to see the macula densa.

The superfusion pipet was then advanced to cover the perfused specimen, and superfusion was started at a rate of 0.6 μl/min. After an equilibration period of 15 minutes, the bath medium was exchanged with water-equilibrated pre-warmed mineral oil. The droplet which formed at the tip of the superfusion pipet was collected at timed intervals (10 min) into a pipet made by pulling a tapered tip on a constant bore micro-capillary (Acupette, Dade Diagnostics, Inc., Aguada, Puerto Rico). This enabled direct volume measurement without transfer. The collected superfusate was frozen for later renin assay.

After the experiment the mineral oil was replaced with fresh Krebs solution, and the specimen was blown off the holding pipet into the bath chamber. It was picked up on a 1.2 mm disc of filter paper using two pieces of closely fitting concentric glass capillary tubing (diameter ca. 1.2 mm). The inner glass capillary was attached to a mouth suction tube. The outer piece of glass tubing was used to punch out a filter from a Millipore SWC4000 filter placed on a pad of nitrile rubber. The inner capillary was then advanced to the filter, thus sealing the circumference of the filter, and acting as a piston keeping the filter at the tip. The specimen was sucked onto the filter. The filter together with the specimen were then expelled into a collection tube by advancing the inner glass tubing. In this way transfer of bath fluid with the specimen was minimized.

Renin released was measured by radioimmunoassay of generated angiotensin I using the "trapping" technique of Poulsen and Jörgensen [9], as modified later [10] for ultramicro-RIA. Renin is expressed in terms of standard Goldblatt hog units (GU) by comparison with standard renin obtained from the Institute for Medical Research (M.R.C., Holly Hill, London, UK). The detection limit of the assay was 0.6 nanoGU. The renin remaining in the specimens after the experiments was extracted by freezing and thawing four times.

A Krebs bicarbonate buffer of the following composition was used (mmol/liter): NaCl 115, $HaHCO_3$ 25, NaAcetate 10, NaH_2PO_4 0.96, Na_2HPO_4 0.24, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 1.0, glucose 5.5, giving a total measured osmolality of 300 mOsm/kg. The solutions were bubbled with 5% CO_2 and 95% O_2 to a pH of 7.4. When used as dissection medium, 2% fetal calf serum was added. In the bath solution used for superfusion 0.3% human albumin was present. This solution was used without albumin as the tubular perfusate in control periods. The solutions used in the perfusion apparatus were bubbled at 37°C in order to avoid formation of bubbles in the glass pipets.

The superfusion pipet is filled with freshly bubbled fluid immediately prior to each study. The fluid in the pipet acts as a reservoir (volume approximately 400 μl) for superfusion throughout the study. Due to the low superfusion speed, only 20% to 40% of the volume present in the superfusion pipet is delivered during an experiment. The CO_2 tension in the buffer was measured to be 21 mm Hg immediately after bubbling, and 23 mm Hg in the superfusion pipette after three hours of superfusion. The pH was 7.49 and 7.42.

With the distal end of the tubule open, the collected fluid contains both superfusate and tubular perfusate; at rates used in the present studies, the ratio is approximately 60:1. By the use of a perfusate containing ^3H inulin, perfusion speed was calibrated by measuring the rate of appearance of inulin in the collections. The hydraulic pressure head was adjusted to maintain a perfusion rate of approximately 10 nl/min. Exchange of perfusate with emptying of the tip dead space in perfusion and superfusion pipets was complete in less than two minutes.

Temperature was maintained by convection of heat into the superfusion chamber. The rate of heat dissipation is dependent on superfusate speed. The temperature was measured by introducing a second temperature-sensor into the superfusion pipet at the site where the isolated perfused tubule usually sits. At a superfusion speed of 0.6 μl per min, the temperature at this site was found to be held at 37.2°C by convection of heat from the oil bath when the bath was controlled to a temperature of 40°C. Phasic fluctuations in the temperature due to feedback control had an amplitude of less than 0.3°C.

Results

Figure 2 shows results from 10 nephrons perfused under basal conditions for 130 minutes. Collections were made every ten minutes. The release rate declined somewhat between the first and second periods and was stable thereafter over this period of observation. Basal renin secretion rate averaged 1.81 nGU per minute per JGA (arithmetic mean) or 0.9 nGU per minute per JGA (geometric mean), with a range of 0.12 to 8.73 nGU per minute per JGA. Figure 3 shows the results of a single experiment in which perfusate composition was changed after 50 minutes of perfusion. In response to a change in tubular fluid

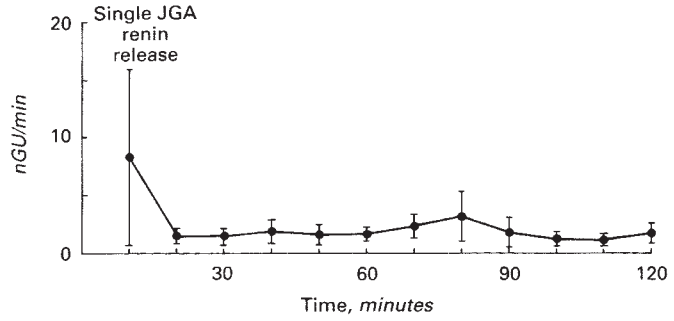

Fig. 2. *The time course of renin secretion from 10 juxtaglomerular apparatuses that were perfused with control solution throughout the experiments.* After 50 minutes a mock perfusate change was made. Bars indicate SE. Data represents 10 experiments, 6 of which were included in the control series from (5).

composition, a prompt increase could be detected in the amount of renin released into the superfusate. The stimulation was already apparent in the first collection made after the perfusate change.

The renin content from 34 microdissected and perfused juxtaglomerular apparatuses was determined. The values were not normally distributed, but a log transformation was found to produce homogeneity of variance by the Bartlett test (Systat statistical program). Arithmetic mean renin content was 349 ± 111 and geometric mean was 45.65 μGU per JGA. The arithmetic mean of the secretion rates was 2.96 ± 1.27 nGU per JGA per min, the geometric mean was 1.50 nGU per JGA per min. The mean fractional release rate was 0.78 ± 0.23% per hour.

Discussion

In this study we present a simple modification of a commercially available microperfusion apparatus which allows the simultaneous superfusion and perfusion of the isolated specimen. Other microperfusion systems could be modified as well, since the only prerequisite is a holder and track system which takes a third concentric pipet in front of the standard holding and perfusion pipets. The front pipet holder was originally designed to hold a Sylgard filled pipet for electrical isolation. The only extra piece of equipment necessary for the modification is a superfusion pump; we use a simple syringe pump with an adaptor to use a glass microliter syringe. The superfusion pipet is easily made with standard techniques.

There were several reasons for developing a superfusion system instead of timed exchanges of bath fluid. Interruption of bath flow to allow accumulation of renin in the bath chamber would result in variable conditions with regard to substrate delivery, mixing and potential unstirred layer effects. If renin were allowed to accumulate in the juxtaglomerular area this might give rise to production of angiotensin II, which could inhibit the secretion rate. Accumulation of other metabolic products could interfere as well, and insufficient oxygenation could become a problem. Exchange of bath fluid from the chamber is associated with a risk of damaging the segment or dislodging it from the holding pipet [2]. The renin release process is known to be sensitive to physical agitation [11]. With the superfusion method all the secreted product is collected. This is of advantage when the amount of product is close to the

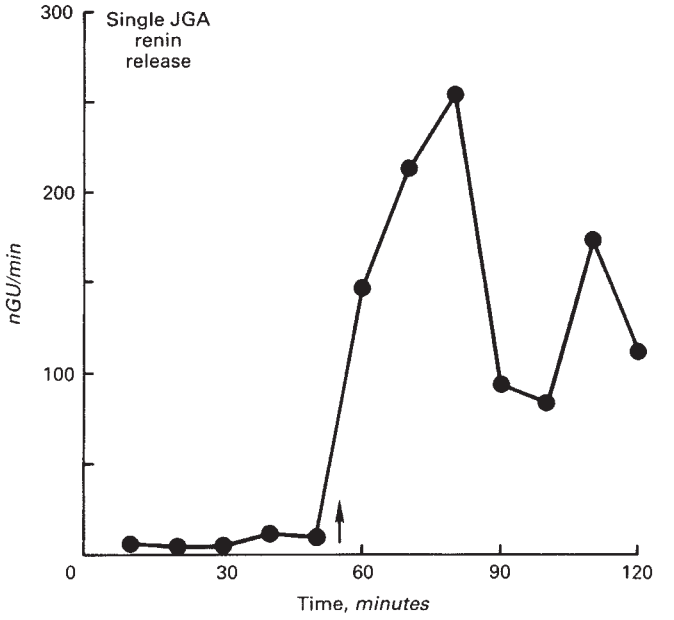

Fig. 3. *The time course of renin secretion from a juxtaglomerular apparatus perfused with control solution for the initial 50 minutes after which the perfusate was changed to an identical solution but with NaCl concentration reduced by 95 mM.* Osmolarity was maintained constant by addition of mannitol.

detection limits of the assay, as was sometimes the case in the basal secretion periods. Because quantitative collections were made, it was possible to calculate single JGA renin release rate. Under basal conditions in these experiments the JGA releases only a very small fraction of its renin content per hour (0.8%). This value is lower than values previously measured from superfused rat arterioles (1.8%) [12] or incubated rabbit arterioles (2.8%) [13], and (1.7%) [14]. This may be due to an inhibitory effect of the macula densa on the renin release rate. Itoh and Carretereo demonstrated an inhibitory effect of the presence of the macula densa. They observed the fractional release rate was 0.25% per hour when the macula densa was thought to be present, whereas the fractional release rate was 1.7% in its absence [14].

The present technique allows simultaneous microperfusion and collection for measuring any product of interest. The limiting factor is the availability of sensitive assays. We have taken advantage of a very sensitive RIA to measure the response of renin secretion to variations in tubular fluid composition. Other assays could be applied; for example an ELISA assay for prostaglandins has recently been shown to have sufficient sensitivity to measure production by a single glomerulus [15].

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